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(54) Title: METHOD OF TREATING DEMYELINATING INFLAMMATORY DISEASE USING CCR1 ANTAGONISTS

(57) Abstract: The invention relates to a method for treating inflammatory demyelinating diseases. The method comprises adminis-
tering to a subject in need an effective amount of an antagonist of CCR1 function. In a preferred embodiment, the invention provides
a method for treating multiple sclerosis.

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B. FIELDS SEARCHED

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 56771 A (SCHERING AG) 17 December 1998 (1998-12-17) * see in particular claims 31,33,41,43; pages 1-4, 35 (line 17) - 37 (line 6); examples 24-26 (pages 220-224) *	1-5,7-11
X	WO 98 38167 A (BROWN MATTHEW FRANK ;KATH JOHN CHARLES (US); PFIZER (US); POSS CHR) 3 September 1998 (1998-09-03) cited in the application *see in particular claims 41-46; page 1, lines 9-24; page 49, line 18 - page 52, lines 26*	1-3,5, 7-11,17, 18, 20-22,26

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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INSERT B.

METHOD OF TREATING DEMYELINATING INFLAMMATORY DISEASE USING CCR1 ANTAGONISTS

RELATED APPLICATION

This application is a continuation-in-part of U.S. Serial No. 09/240,253, filed
5 January 29, 1999, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The inflammatory demyelinating diseases share the common features of inflammation (e.g., leukocyte infiltration) and destruction of the myelin of the central nervous system (CNS). The pathophysiology of these diseases involves
10 infiltration of the CNS by leukocytes (e.g., T cells, macrophages) and the subsequent development of plaques, which are areas of demyelination. These diseases can be acute (e.g., acute disseminated encephalomyelitis, Guillain-Barre syndrome and acute hemorrhagic leukoencephalitis) or chronic (e.g., multiple sclerosis, chronic inflammatory demyelinating polyradiculoneuropathy). No specific diagnostic test
15 exists for the demyelinating diseases and diagnosis is usually based upon recognition of the distinctive pattern of CNS injury each disease produces.

The most common demyelinating disease is multiple sclerosis (MS) which affects about 350,000 Americans, and with the exception of trauma, is the most frequent cause of neurological disability commencing in early adulthood to middle
20 age. The manifestation of MS is highly variable and the disease can be benign or rapidly debilitating. The clinical course of MS can be grouped into four general categories. Relapsing-remitting MS is characterized by recurrent acute attacks of neurological dysfunction followed by periods when recovery may occur. No progression of neurological impairment occurs between attacks. Secondary
25 progressive MS has a relapsing-remitting course at first, but later, neurological dysfunction progresses (i.e., becomes more severe) during the period between acute attacks. Primary progressive MS is characterized by a progression of disability from the onset of disease with no distinct acute attacks. Some patients with primary

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progressive MS can experience periods of apparent clinical stability. The most rare course is referred to as progressive-relapsing MS which is characterized by a progression of disability from the onset of disease with recurrent acute attacks.

Insights into the pathogenesis of the demyelinating diseases have been
5 derived from studying experimental allergic encephalomyelitis (EAE) in mice and rats. EAE is an inflammatory demyelinating disease mediated by CD4⁺ Th1 cells which serves as a model for MS. EAE, MS and other demyelinating diseases appear to be mediated by an autoimmune mechanism which involves the activation and recruitment of lymphocytes (e.g., T cells) that are reactive to certain self-antigens
10 expressed in the CNS. Furthermore, there is some evidence for a genetic predisposition for developing demyelinating disease and environmental factors (e.g., viral infection) may trigger the onset of disease.

Treatment of the demyelinating disease is primarily focused on inhibiting acute attacks with adrenocorticotrophic hormone (ACTH) or immunosuppressive
15 agents (e.g., glucocorticoids). These agents can produce severe systemic side effects and, thus, only moderate and severe attacks are usually treated. Interferons (e.g., IFN β -1a, IFN β -1b) and copolymer 1 have been used for prophylaxis against recurrent acute attacks (relapse) in MS. However, the efficacy of these therapies is often limited by systemic reactions (e.g., neutralizing antibodies) to the drugs.
20 Additionally, chronic immunosuppression (e.g., with methotrexate, azathioprene, cyclophosphamide, 2-chlorodeoxyadenosine) has been used with only modest efficacy for the treatment of progressive MS.

Thus, a need exists for an efficacious method of treating demyelinating diseases.

25 SUMMARY OF THE INVENTION

The invention relates to a method for treating inflammatory demyelinating diseases. The method comprises administering an effective amount of an antagonist of CCR1 function to a subject in need thereof.

In one aspect, the invention provides a method of treating an inflammatory
30 demyelinating disease comprising administering to a subject in need thereof an effective amount of an (i.e., one or more) antagonist of CCR1 function.

In one embodiment, the method is directed toward treating an acute inflammatory demyelinating disease, for example, acute disseminated encephalomyelitis, Guillain-Barre syndrome or acute hemorrhagic leukoencephalitis. In another embodiment, the method is directed toward treating a chronic inflammatory disease, for example, MS or chronic inflammatory demyelinating polyradiculoneuropathy. In a preferred embodiment, the invention provides a method of treating MS.

The antagonist of CCR1 function is a molecule, such as, a protein, peptide, peptidomimetic, natural product or small organic molecule, that inhibits (reduces, prevents) one or more functions of CCR1.

In another aspect, the invention provides a method of treating an inflammatory demyelinating disease comprising administering to a subject in need thereof an effective amount of an antagonist of CCR1 function and an effective amount of one or more additional therapeutic agents, such as, antiviral, antibacterial and immunosuppressive agents.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a histogram illustrating the clinical scores of young female CCR1 knockout mice (CCR1 KO, n=10; solid bars) and age and sex matched wild type control mice (B6/129, n=10; open bars) at various times following immunization with MOG(35-55) peptide in Freund's complete adjuvant (CFA) supplemented with mycobacterium tuberculosis antigen (4 mg/ml). Clinical scores shown are the mean daily score for each group.

Figure 2 is a histogram illustrating the clinical scores of young female CCR1 knockout mice (CCR1 KO, n=8; solid bars) and age and sex matched wild type control mice (B6/129, n=8; open bars) at various times following immunization with MOG(35-55) peptide in Freund's complete adjuvant (CFA) supplemented with mycobacterium tuberculosis antigen (4 mg/ml). Clinical scores shown are the mean daily score for each group.

Figure 3 is a histogram illustrating the clinical scores of adult male CCR1 knockout mice (CCR1 KO, n=10; solid bars) and age and sex matched wild type control mice (B6/129, n=10; open bars) at various times following immunization

with MOG(35-55) peptide in Freund's complete adjuvant (CFA) supplemented with mycobacterium tuberculosis antigen (4 mg/ml). Clinical scores shown are the mean daily score for each group.

Figure 4 is a histogram illustrating the change in ear thickness associated with a delayed type hypersensitivity response in young female CCR1 KO mice (n=8) and age and sex matched wild type control mice (n=8) that were sensitized and challenged with 0.5% 2,4-dinitrofluorobenzene (DNFB).

Figure 5A is a graph illustrating dose-dependent proliferation of splenocytes in an *in vitro* proliferation assay. Splenocytes isolated from CCR1 $-/-$ mice or wild type B6/129 mice (CCR1 $+/+$) were cultured with MOG(35-55) peptide. Proliferation was detected by pulsing the cultures with ^3H -thymidine and quantifying the amount of radioactivity incorporated into the DNA of cells.

Figure 5B is a graph illustrating dose-dependent production of IL-2 in cultures of splenocytes stimulated with MOG(35-55) peptide. Splenocytes isolated from CCR1 $-/-$ mice or wild type B6/129 mice (CCR1 $+/+$) were cultured in serum-free media containing MOG(35-55) peptide for 40 hours. The culture supernatants were removed and the amount of IL-2 in the supernatants was determined by quantitative ELISA.

Figure 5C is a graph illustrating dose-dependent production of IFN- γ in cultures of splenocytes stimulated with MOG(35-55) peptide. Splenocytes isolated from CCR1 $-/-$ mice or wild type B6/129 mice (CCR1 $+/+$) were cultured in serum-free media containing MOG(35-55) peptide for 40 hours. The culture supernatants were removed and the amount of IFN γ in the supernatants was determined by quantitative ELISA.

Figure 5D is a graph illustrating dose-dependent production of IL-6 in cultures of splenocytes stimulated with MOG(35-55) peptide. Splenocytes isolated from CCR1 $-/-$ mice or wild type B6/129 mice (CCR1 $+/+$) were cultured in serum-free media containing MOG(35-55) peptide for 40 hours. The culture supernatants were removed and the amount of IL-6 in the supernatants was determined by quantitative ELISA.

DETAILED DESCRIPTION OF THE INVENTION

Chemokines are a family of proinflammatory mediators that promote recruitment and activation of multiple lineages of leukocytes (e.g., lymphocytes, macrophages). They can be released by many kinds of tissue cells after activation.

- 5 Continuous release of chemokines at sites of inflammation can mediate the ongoing migration and recruitment of effector cells to sites of chronic inflammation. The chemokines are related in primary structure and share four conserved cysteines, which form disulfide bonds. Based upon this conserved cysteine motif, the family can be divided into distinct branches, including the C-X-C chemokines
- 10 (α -chemokines), and the C-C chemokines (β -chemokines), in which the first two conserved cysteines are separated by an intervening residue, or are adjacent residues, respectively (Baggiolini, M. and Dahinden, C. A., *Immunology Today*, 15:127-133 (1994)).

- The C-X-C chemokines include a number of potent chemoattractants and
- 15 activators of neutrophils, such as interleukin 8 (IL-8), PF4 and neutrophil-activating peptide-2 (NAP-2). The C-C chemokines include, for example, RANTES (Regulated on Activation, Normal T Expressed and Secreted), the macrophage inflammatory proteins 1 α and 1 β (MIP-1 α and MIP-1 β), eotaxin and human monocyte chemotactic proteins 1-3 (MCP-1, MCP-2, MCP-3), which have been
- 20 characterized as chemoattractants and activators of monocytes or lymphocytes. Chemokines, such as RANTES and MIP-1 α , have been implicated in human acute and chronic inflammatory diseases including respiratory diseases, such as asthma and allergic disorders.

- The chemokine receptors are members of a superfamily of G protein-coupled
- 25 receptors (GPCR) which share structural features that reflect a common mechanism of action of signal transduction (Gerard, C. and Gerard, N.P., *Annu Rev. Immunol.*, 12:775-808 (1994); Gerard, C. and Gerard, N. P., *Curr. Opin. Immunol.*, 6:140-145 (1994)). Conserved features include seven hydrophobic domains spanning the plasma membrane, which are connected by hydrophilic extracellular and
- 30 intracellular loops. The majority of the primary sequence homology occurs in the hydrophobic transmembrane regions with the hydrophilic regions being more

diverse. The receptors for the C-C chemokines include: CCR1 which can bind, for example, MIP-1 α , RANTES, MCP-2, MCP-3, MCP-4, CKbeta8, CKbeta8-1, leukotactin-1, HCC-1 and MPIF-1; CCR2 which can bind, for example, MCP-1, MCP-2, MCP-3 and MCP-4; CCR3 which can bind, for example, eotaxin, eotaxin-2, 5 RANTES, MCP-2, MCP-3 and MCP-4; CCR4 which can bind, for example, TARC, RANTES, MIP-1 α and MCP-1; CCR5 which can bind, for example, MIP-1 α , RANTES, and MIP-1 β ; CCR6 which can bind, for example, LARC/MIP-3 α /exodus; CCR7 which can bind, for example, ELC/MIP-3 β and CCR8 which can bind, for example I-309 (Baggiolini, M., *Nature* 392:565-568 (1998); Luster, A.D., 10 *New England Journal of Medicine*, 338(7):436-445 (1998); Tsou, *et al.*, *J. Exp. Med.*, 188:603-608 (1998); Nardelli, *et al.*, *J Immunol*, 162(1):435-444 (1999); Youn, *et al.*, *Blood*, 91(9):3118-3126 (1998); Youn, *et al.*, *J Immunol*, 159(11):5201-5201 (1997)).

The human chemokine receptor CCR1 is expressed on a variety of different 15 cells including neutrophils, monocytes, lymphocytes, and eosinophils, and CCR1 binds a variety of chemokine ligands (Gong, X. *et al.*, *J. Biol. Chem.*, 272: 11682-11685 (1997); Wong, M. and E.N. Fish, *J. Biol. Chem.*, 273: 309-341 (1998); and Youn, B.S. *et al.*, *J. Immunol.*, 159: 5201-5205 (1997)).

To determine whether CCR1 function is involved in the initiation, progression 20 and/or maintenance of demyelinating lesions in the CNS, studies of EAE, the cellular immunity-mediated (e.g., Th1-mediated) autoimmune disease that is a model of MS, in wild type and genetically altered mice were undertaken. As described herein, neurological dysfunction, CNS inflammation and CNS myelin destruction, which are hallmarks of EAE and MS, were dramatically inhibited (e.g., 25 reduced or prevented) in CCR1^{-/-} mice as compared to age and sex matched CCR1^{+/+} control mice.

The incidence of EAE, like MS, is influenced by gender, and female mice develop disease more frequently and with greater severity than do male mice. Consequently, EAE is usually studied in young (e.g., 6-9 week old) female mice.

30 As described herein, EAE was induced in age and sex matched CCR1^{-/-} and CCR1^{+/+} mice by immunization with an immunodominant epitope of myelin oligodendrocyte glycoprotein (MOG(35-55); Hilton, A.A., *et al. J. Neurochem.*,

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65:309-318 (1995)), and the onset, course and severity of disease was assessed by monitoring neurological dysfunction (e.g., tail tone, posterior paresis, loss of righting response, tetraparesis).

5 Young female CCR1 $-/-$ mice developed EAE that was significantly less severe than the disease in CCR1 $+/+$ control animals. Furthermore, the young female CCR1 $-/-$ mice had a decreased incidence of disease, and the onset of clinical disease (e.g., neurological dysfunction) was delayed in comparison with control CCR1 $+/+$ mice. (Figures 1 and 2; Example 1, Experiments 1 and 2)

10 In another study employing adult male mice, 80% of the CCR1 $+/+$ control mice developed EAE. In contrast, CCR1 $-/-$ mice had a significantly decreased incidence of disease, with only one of ten animals developing EAE. The single CCR $-/-$ mouse that developed EAE had only minimal clinical symptoms that were dramatically less severe than the clinical symptoms of CCR1 $+/+$ control mice. (Figure 3; Example 1, Experiment 3).

15 Histological examination of brains and spinal cords removed from mice immunized with MOG(35-55) revealed a significant reduction in the degree of inflammation and myelin destruction in the CNS of CCR1 $-/-$ mice ($n=3$) compared with CCR1 $+/+$ control mice ($n=4$) (Example 1, Microscopic examination). Thus, CCR1 $-/-$ mice develop less severe clinical symptoms, in comparison with CCR1
20 $+/+$ control mice, because disruption of CCR1 function inhibits CNS inflammation.

Further studies demonstrated that the remarkable decrease in severity and incidence of EAE-associated pathology in CCR1 $-/-$ mice is not due to a general defect in cellular immunity in these animals. In fact, CCR1 $-/-$ mice have the capacity to mount a normal cellular immune response as assessed by a mixed
25 lymphocyte reaction (MLR), and unsensitized CCR1 $-/-$ mice are hyper-responsive in a standard delayed type hypersensitivity (DTH) assay (Example 2, Figure 4). Thus, CCR1 function is specifically involved in the pathogenesis of EAE, and disruption of CCR1 function inhibits (reduces or prevents) EAE-associated neurological dysfunction, CNS inflammation and CNS myelin destruction in
30 CCR1 $-/-$ mice.

Accordingly, a first aspect of the invention provides a method for treating an inflammatory demyelinating disease comprising administering to a subject in need thereof an effective amount of an antagonist of CCR1 function.

CCR1 antagonists

- 5 As used herein, the term "antagonist of CCR1 function" refers to an agent (e.g., a molecule, a compound) which can inhibit a (i.e., one or more) function of CCR1. For example, an antagonist of CCR1 function can inhibit the binding of one or more ligands (e.g., MIP-1 α , RANTES, MCP-2, MCP-3, MCP-4, CKbeta8, CKbeta8-1, leukotactin-1, HCC-1, MPIF-1) to CCR1 and/or inhibit signal
- 10 transduction mediated through CCR1 (e.g., GDP/GTP exchange by CCR1 associated G proteins, intracellular calcium flux). Accordingly, CCR1-mediated processes and cellular responses (e.g., proliferation, migration, chemotactic responses, secretion or degranulation) can be inhibited with an antagonist of CCR1 function.
- 15 Preferably, the antagonist of CCR1 function is a compound which is, for example, a small organic molecule, natural product, protein (e.g., antibody, chemokine, cytokine), peptide or peptidomimetic. Several molecules that can antagonize one or more functions of chemokine receptors (e.g., CCR1) are known in the art, including the small organic molecules disclosed in, for example,
- 20 international patent application WO 97/24325 by Takeda Chemical Industries, Ltd.; WO 98/38167 by Pfizer, Inc.; WO 97/44329 by Teijin Limited; WO 98/04554 by Banyu Pharmaceutical Co., Ltd.; WO 98/27815, WO 98/25604, WO 98/25605, WO 98/25617 and WO 98/31364 by Merck & Co., Inc.; WO 98/02151 and WO 99/37617, by LeukoSite, Inc.; WO 99/37651 and WO 99/37619 by LeukoSite, Inc.,
- 25 *et al.*; United States Provisional Patent Application Number 60/021,716, filed July 12, 1996; United States Patent Application Numbers: 09/146,827 and 09/148,236, filed September 4, 1998; Hesselgesser *et al.*, *J. Biol. Chem.* 273(25):15687-15692 (1998); and Howard *et al.*, *J. Medicinal Chem.* 41(13):2184-2193 (1998); proteins, such as antibodies (e.g., polyclonal sera, monoclonal, chimeric, humanized) and
- 30 antigen-binding fragments thereof (e.g., Fab, Fab', F(ab')₂, Fv), for example, those disclosed in Su *et al.*, *J. Leukocyte Biol.* 60:658-656 (1996); chemokine mutants and

analogues, for example, those disclosed in U.S. Patent No. 5,739,103 issued to Rollins *et al.*, WO 96/38559 by Dana Farber Cancer Institute and WO 98/06751 by Research Corporation Technologies, Inc.; peptides, for example, those disclosed in WO 98/09642 by The United States of America. The entire teachings of each of the
5 above cited patent applications and references is incorporated herein by reference.

Antagonists of CCR1 function can be identified, for example, by screening libraries or collections of molecules, such as, the Chemical Repository of the National Cancer Institute (U.S.A.), as described herein or using other suitable methods. Antagonists thus identified can be used in the therapeutic methods
10 described herein.

Another source of antagonists of CCR1 function are combinatorial libraries which can comprise many structurally distinct molecular species. Combinatorial libraries can be used to identify lead compounds or to optimize a previously identified lead. Such libraries can be manufactured by well-known methods of
15 combinatorial chemistry and screened by suitable methods, such as the methods described herein.

The term "natural product", as used herein, refers to a compound which can be found in nature, for example, naturally occurring metabolites of marine organisms (e.g., tunicates, algae) and plants and which possess biological activity, e.g., can
20 antagonize CCR1 function. For example, lactacystin, paclitaxel and cyclosporin A are natural products which can be used as anti-proliferative or immunosuppressive agents.

Natural products can be isolated and identified by suitable means. For example, a suitable biological source (e.g., vegetation) can be homogenized (e.g., by
25 grinding) in a suitable buffer and clarified by centrifugation, thereby producing an extract. The resulting extract can be assayed for the capacity to antagonize CCR1 function, for example, by the assays described herein. Extracts which contain an activity that antagonizes CCR1 function can be further processed to isolate the CCR1 antagonist by suitable methods, such as, fractionation (e.g., column
30 chromatography (e.g., ion exchange, reverse phase, affinity), phase partitioning, fractional crystallization) and assaying for biological activity (e.g., antagonism of CCR1 activity). Once isolated the structure of a natural product can be determined

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(e.g., by nuclear magnetic resonance (NMR)) and those of skill in the art can devise a synthetic scheme for synthesizing the natural product. Thus, a natural product can be isolated (e.g., substantially purified) from nature or can be fully or partially synthetic. A natural product can be modified (e.g., derivatized) to optimize its
5 therapeutic potential. Thus, the term "natural product", as used herein, includes those compounds which are produced using standard medicinal chemistry techniques to optimize the therapeutic potential of a compound which can be isolated from nature.

The term "peptide", as used herein, refers to a compound consisting of from
10 about two to about ninety amino acid residues wherein the amino group of one amino acid is linked to the carboxyl group of another amino acid by a peptide bond. A peptide can be, for example, derived or removed from a native protein by enzymatic or chemical cleavage, or can be prepared using conventional peptide synthesis techniques (e.g., solid phase synthesis) or molecular biology techniques
15 (see Sambrook, J. *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). A "peptide" can comprise any suitable L- and/or D-amino acid, for example, common α -amino acids (e.g., alanine, glycine, valine), non- α -amino acids (e.g., β -alanine, 4-aminobutyric acid, 6-aminocaproic acid, sarcosine, statine), and unusual amino acids (e.g., citrulline,
20 homocitrulline, homoserine, norleucine, norvaline, ornithine). The amino, carboxyl and/or other functional groups on a peptide can be free (e.g., unmodified) or protected with a suitable protecting group. Suitable protecting groups for amino and carboxyl groups, and means for adding or removing protecting groups are known in the art and are disclosed in, for example, Green and Wuts, "*Protecting Groups in*
25 *Organic Synthesis*", John Wiley and Sons, 1991. The functional groups of a peptide can also be derivatized (e.g., alkylated) using art-known methods.

Peptides can be synthesized and assembled into libraries comprising a few to many discrete molecular species. Such libraries can be prepared using well-known methods of combinatorial chemistry, and can be screened as described herein or
30 using other suitable methods to determine if the library comprises peptides which can antagonize CCR1 function. Such peptide antagonists can then be isolated by suitable methods.

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The term "peptidomimetic", as used herein, refers to molecules which are not polypeptides, but which mimic aspects of their structures. For example, polysaccharides can be prepared that have the same functional groups as peptides which can antagonize CCR1. Peptidomimetics can be designed, for example, by
5 establishing the three dimensional structure of a peptide agent in the environment in which it is bound or will bind to CCR1. The peptidomimetic comprises at least two components, the binding moiety or moieties and the backbone or supporting structure.

The binding moieties are the chemical atoms or groups which will react or
10 form a complex (e.g., through hydrophobic or ionic interactions) with CCR1, for example, with the amino acid(s) at or near the ligand binding site. For example, the binding moieties in a peptidomimetic can be the same as those in a peptide antagonist of CCR1. The binding moieties can be an atom or chemical group which reacts with the receptor in the same or similar manner as the binding moiety in a
15 peptide antagonist of CCR1. Examples of binding moieties suitable for use in designing a peptidomimetic for a basic amino acid in a peptide are nitrogen containing groups, such as amines, ammoniums, guanidines and amides or phosphoniums. Examples of binding moieties suitable for use in designing a peptidomimetic for an acidic amino acid can be, for example, carboxyl, lower alkyl
20 carboxylic acid ester, sulfonic acid, a lower alkyl sulfonic acid ester or a phosphorous acid or ester thereof.

The supporting structure is the chemical entity that, when bound to the binding moiety or moieties, provides the three dimensional configuration of the peptidomimetic. The supporting structure can be organic or inorganic. Examples of
25 organic supporting structures include polysaccharides, polymers or oligomers of organic synthetic polymers (such as, polyvinyl alcohol or polylactide). It is preferred that the supporting structure possess substantially the same size and dimensions as the peptide backbone or supporting structure. This can be determined by calculating or measuring the size of the atoms and bonds of the peptide and
30 peptidomimetic. In one embodiment, the nitrogen of the peptide bond can be substituted with oxygen or sulfur, thereby forming a polyester backbone. In another embodiment, the carbonyl can be substituted with a sulfonyl group or sulfinyl group,

thereby forming a polyamide (e.g., a polysulfonamide). Reverse amides of the peptide can be made (e.g., substituting one or more -CONH- groups for a -NHCO- group). In yet another embodiment, the peptide backbone can be substituted with a polysilane backbone.

5 These compounds can be manufactured by known methods. For example, a polyester peptidomimetic can be prepared by substituting a hydroxyl group for the corresponding α -amino group on amino acids, thereby preparing a hydroxyacid and sequentially esterifying the hydroxyacids, optionally blocking the basic and acidic side chains to minimize side reactions. Determining an appropriate chemical
10 synthesis route can generally be readily identified upon determining the chemical structure.

Peptidomimetics can be synthesized and assembled into libraries comprising a few to many discrete molecular species. Such libraries can be prepared using well-known methods of combinatorial chemistry, and can be screened as described herein
15 to determine if the library comprises one or more peptidomimetics which antagonize CCR1 function. Such peptidomimetic antagonists can then be isolated by suitable methods.

In one embodiment, the CCR1 antagonist is an antibody or antigen-binding fragment thereof having specificity for CCR1. The antibody can be polyclonal or
20 monoclonal, and the term "antibody" is intended to encompass both polyclonal and monoclonal antibodies. The terms polyclonal and monoclonal refer to the degree of homogeneity of an antibody preparation, and are not intended to be limited to particular methods of production. The term "antibody" as used herein also encompasses functional fragments of antibodies, including fragments of chimeric,
25 humanized, primatized, veneered or single chain antibodies. Functional fragments include antigen-binding fragments which bind to CCR1. For example, antibody fragments capable of binding to CCR1 or portions thereof, including, but not limited to Fv, Fab, Fab' and F(ab')₂ fragments are encompassed by the invention. Such fragments can be produced by enzymatic cleavage or by recombinant techniques.
30 For example, papain or pepsin cleavage can generate Fab or F(ab')₂ fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or F(ab')₂ fragments. Antibodies can also be produced in a variety

of truncated forms using antibody genes in which one or more stop codons has been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F(ab')₂ heavy chain portion can be designed to include DNA sequences encoding the CH₁ domain and hinge region of the heavy chain. Single chain antibodies, and
5 chimeric, humanized or primatized (CDR-grafted), or veneered antibodies, as well as chimeric, CDR-grafted or veneered single chain antibodies, comprising portions derived from different species, and the like are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by conventional techniques, or can be prepared as a
10 contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly *et al.*, U.S. Patent No. 4,816,567; Cabilly *et al.*, European Patent No. 0,125,023 B1; Boss *et al.*, U.S. Patent No. 4,816,397; Boss *et al.*, European Patent No. 0,120,694 B1; Neuberger, M.S. *et al.*, WO 86/01533;
15 Neuberger, M.S. *et al.*, European Patent No. 0,194,276 B1; Winter, U.S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen *et al.*, European Patent No. 0 451 216 B1; and Padlan, E.A. *et al.*, EP 0 519 596 A1. See also, Newman, R. *et al.*, *BioTechnology*, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner *et al.*, U.S. Patent No. 4,946,778 and Bird, R.E. *et al.*, *Science*,
20 242: 423-426 (1988)) regarding single chain antibodies.

Humanized antibodies can be produced using synthetic or recombinant DNA technology using standard methods or other suitable techniques. Nucleic acid (e.g., cDNA) sequences coding for humanized variable regions can also be constructed using PCR mutagenesis methods to alter DNA sequences encoding a human or
25 humanized chain, such as a DNA template from a previously humanized variable region (see e.g., Kamman, M., *et al.*, *Nucl. Acids Res.*, 17: 5404 (1989)); Sato, K., *et al.*, *Cancer Research*, 53: 851-856 (1993); Daugherty, B.L. *et al.*, *Nucleic Acids Res.*, 19(9): 2471-2476 (1991); and Lewis, A.P. and J.S. Crowe, *Gene*, 101: 297-302 (1991)). Using these or other suitable methods, variants can also be readily
30 produced. In one embodiment, cloned variable regions can be mutated, and sequences encoding variants with the desired specificity can be selected (e.g., from a

phage library; see e.g., Krebber *et al.*, U.S. 5,514,548; Hoogenboom *et al.*, WO 93/06213, published April 1, 1993).

Antibodies which are specific for mammalian (e.g., human) CCR1 can be raised against an appropriate immunogen, such as isolated and/or recombinant human CCR1 or portions thereof (including synthetic molecules, such as synthetic peptides). Antibodies can also be raised by immunizing a suitable host (e.g., mouse) with cells that express CCR1, such as activated T cells (see e.g., U.S. Pat. No. 5,440,020, the entire teachings of which are incorporated herein by reference). In addition, cells expressing recombinant CCR1 such as transfected cells, can be used as immunogens or in a screen for antibody which binds receptor (See e.g., Chuntharapai *et al.*, *J. Immunol.*, 152: 1783-1789 (1994); Chuntharapai *et al.*, U.S. Patent No. 5,440,021).

Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed using any suitable technique. A variety of methods have been described (see e.g., Kohler *et al.*, *Nature*, 256: 495-497 (1975) and *Eur. J. Immunol.* 6: 511-519 (1976); Milstein *et al.*, *Nature* 266: 550-552 (1977); Koprowski *et al.*, U.S. Patent No. 4,172,124; Harlow, E. and D. Lane, 1988, *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory: Cold Spring Harbor, NY); *Current Protocols In Molecular Biology*, Vol. 2 (Supplement 27, Summer '94), Ausubel, F.M. *et al.*, Eds., (John Wiley & Sons: New York, NY), Chapter 11, (1991)). Generally, a hybridoma is produced by fusing a suitable immortal cell line (e.g., a myeloma cell line such as SP2/0 or P3X63Ag8.653) with antibody producing cells. The antibody producing cells, preferably those obtained from the spleen or lymph nodes, can be obtained from animals immunized with the antigen of interest. The fused cells (hybridomas) can be isolated using selective culture conditions, and cloned by limiting dilution. Cells which produce antibodies with the desired specificity can be selected by a suitable assay (e.g., ELISA).

Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library (e.g., a phage display library), or which rely upon immunization of transgenic animals (e.g., mice) capable of producing a repertoire of human antibodies (see e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 2551-

2555 (1993); Jakobovits *et al.*, *Nature*, 362: 255-258 (1993); Lonberg *et al.*, U.S. Patent No. 5,545,806; Surani *et al.*, U.S. Patent No. 5,545,807; Lonberg *et al.*, WO97/13852).

In one embodiment, the antibody or antigen-binding fragment thereof has
5 specificity for a mammalian CC chemokine receptor-1 (CCR1), such as human CCR1. In a preferred embodiment, the antibody or antigen-binding fragment can inhibit binding of a ligand (i.e., one or more ligands) to CCR1 and/or one or more functions mediated by CCR1 in response to ligand binding. Preferred antibody antagonists of CCR1 function are disclosed in our co-pending United States Patent
10 Application titled "Anti-CCR1 Antibodies and Methods of Use Therefor", by Shixin Qin, Walter Newman and Nasim Kassam, Attorney's docket number LKS97-13, U.S. Serial No. 09/239,938, filed January 29, 1999, and in International Application No. PCT/US99/04527, the teachings of each of these applications are incorporated herein by reference in their entirety.

15 Assessment of Activity of Antagonists

The capacity of an agent (e.g., proteins, peptides, natural products, small organic molecules, peptidomimetics) to antagonize CCR1 function can be determined using a suitable screen (e.g., high through-put assay). For example, an agent can be tested in an extracellular acidification assay, calcium flux assay, ligand
20 binding assay or chemotaxis assay (see, for example, Hesselgesser *et al.*, *J. Biol. Chem.* 273(25):15687-15692 (1998) and WO 98/02151).

In a particular assay, membranes can be prepared from cells which express CCR1, such as THP-1 cells (American Type Culture Collection, Manassas, VA; Accession No. TIB202). Cells can be harvested by centrifugation, washed twice
25 with PBS (phosphate-buffered saline), and the resulting cell pellets frozen at -70 to -85°C. The frozen pellet can be thawed in ice-cold lysis buffer consisting of 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) pH 7.5, 2 mM EDTA (ethylenediaminetetraacetic acid), 5 µg/ml each aprotinin, leupeptin, and chymostatin (protease inhibitors), and 100 µg/ml PMSF (phenyl methane sulfonyl
30 fluoride - also a protease inhibitor), at a concentration of 1 to 5 x 10⁷ cells/ml, to achieve cell lysis. The resulting suspension can be mixed well to resuspend all of

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the frozen cell pellet. Nuclei and cell debris can be removed by centrifugation of 400 x g for 10 minutes at 4°C. The resulting supernatant can be transferred to a fresh tube and the membrane fragments can be collected by centrifugation at 25,000 x g for 30 minutes at 4°C. The resulting supernatant can be aspirated and the pellet
5 can be resuspended in freezing buffer consisting of 10 mM HEPES pH 7.5, 300 mM sucrose, 1 µg/ml each aprotinin, leupeptin, and chymostatin, and 10 µg/ml PMSF (approximately 0.1 ml per each 10⁸ cells). All clumps can be resolved using a minihomogenizer, and the total protein concentration can be determined by suitable methods (e.g., Bradford assay, Lowery assay). The membrane solution can be
10 divided into aliquots and frozen at -70 to -85°C until needed.

The membrane preparation described above can be used in a suitable binding assay. For example, membrane protein (2 to 20 µg total membrane protein) can be incubated with 0.1 to 0.2 nM ¹²⁵I-labeled RANTES or MIP-1α with or without unlabeled competitor (RANTES or MIP-1α) or various concentrations of
15 compounds to be tested. ¹²⁵I-labeled RANTES and ¹²⁵I-labeled MIP-1α can be prepared by suitable methods or purchased from commercial vendors (e.g., DuPont-NEN (Boston, MA)). The binding reactions can be performed in 60 to 100 µl of a binding buffer consisting of 10 mM HEPES pH 7.2, 1 mM CaCl₂, 5 mM MgCl₂, and 0.5% BSA (bovine serum albumin), for 60 min at room temperature. The
20 binding reactions can be terminated by harvesting the membranes by rapid filtration through glass fiber filters (e.g., GF/B or GF/C, Packard) which can be presoaked in 0.3% polyethyleneimine. The filters can be rinsed with approximately 600 µl of binding buffer containing 0.5 M NaCl, dried, and the amount of bound radioactivity can be determined by scintillation counting.

25 The CCR1 antagonist activity of test agents (e.g., compounds) can be reported as the inhibitor concentration required for 50% inhibition (IC₅₀ values) of specific binding in receptor binding assays (e.g., using ¹²⁵I-RANTES or ¹²⁵I-MIP-1α as ligand and THP-1 cell membranes). Specific binding is preferably defined as the total binding (e.g., total cpm on filters) minus the non-specific binding. Non-specific
30 binding is defined as the amount of cpm still detected in the presence of excess unlabeled competitor (e.g., RANTES or MIP-1α).

If desired, membranes prepared from cells which express recombinant CCR1 can be used in the described assay.

The capacity of compounds to antagonize CCR1 function can also be determined in a leukocyte chemotaxis assay using suitable cells. Suitable cells include, for example, cell lines, recombinant cells or isolated cells which express CCR1 and undergo CCR1 ligand-induced (e.g., MIP-1 α , RANTES, MCP-2, MCP-3, MCP-4, HCC-1 or MPIF-1) chemotaxis. In one example, CCR1-expressing recombinant L1.2 cells (see Campbell, *et al. J Cell Biol*, 134:255-266 (1996)), peripheral blood mononuclear cells or HL60 cells differentiated with butyric acid, can be used in a modification of a transendothelial migration assay (Carr, M.W., *et al. T.A., Proc. Natl. Acad. Sci., USA*, (91):3652 (1994)). Peripheral blood mononuclear cells can be isolated from whole blood by suitable methods, for example, density gradient centrifugation and positive or preferably negative selection with specific antibodies. The endothelial cells used in this assay are preferably the endothelial cell line, ECV 304, obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.). Endothelial cells can be cultured on 6.5 mm diameter Transwell culture inserts (Costar Corp., Cambridge, MA) with 3.0 μ m pore size. Culture media for the ECV 304 cells can consist of M199+10% FCS, L-glutamine, and antibiotics. The assay media can consist of equal parts RPMI 1640 and M199 with 0.5% BSA. Two hours before the assay, 2×10^5 ECV 304 cells can be plated onto each insert of the 24 well Transwell chemotaxis plate and incubated at 37°C. Chemotactic factors such as RANTES or MIP-1 α (Peprotech)(diluted in assay medium) can be added to the 24-well tissue culture plates in a final volume of 600 μ L. Endothelial-coated Transwells can be inserted into each well and 10^6 cells of the leukocyte type being studied are added to the top chamber in a final volume of 100 μ L of assay medium. The plate can then be incubated at 37°C in 5% CO₂/95% air for 1-2 hours. The cells that migrate to the bottom chamber during incubation can be counted, for example using flow cytometry. To count cells by flow cytometry, 500 μ L of the cell suspension from the lower chamber can be placed in a tube and relative counts can be obtained for a set period of time, for example, 30 seconds. This counting method is highly reproducible and allows gating on the leukocytes and the exclusion of debris or other

cell types from the analysis. Alternatively, cells can be counted with a microscope. Assays to evaluate chemotaxis inhibitors can be performed in the same way as control experiment described above, except that antagonist solutions, in assay media containing up to 1% of DMSO co-solvent, can be added to both the top and bottom chambers prior to addition of the cells. Antagonist potency can be determined by comparing the number of cell that migrate to the bottom chamber in wells which contain antagonist, to the number of cells which migrate to the bottom chamber in control wells. Control wells can contain equivalent amounts of DMSO, but no antagonist.

- 10 The activity of an antagonist of CCR1 function can also be assessed by monitoring cellular responses induced by active receptor, using suitable cells expressing receptor. For instance, exocytosis (e.g., degranulation of cells leading to release of one or more enzymes or other granule components, such as esterases (e.g., serine esterases), perforin, and/or granzymes), inflammatory mediator release (such as release of bioactive lipids such as leukotrienes (e.g., leukotriene C₄)), and respiratory burst, can be monitored by methods known in the art or other suitable methods (see e.g., Taub, D.D. *et al.*, *J. Immunol.*, 155: 3877-3888 (1995), regarding assays for release of granule-derived serine esterases; Loetscher *et al.*, *J. Immunol.*, 156: 322-327 (1996), regarding assays for enzyme and granzyme release; Rot, A. *et al.*, *J. Exp. Med.*, 176: 1489-1495 (1992) regarding respiratory burst; Bischoff, S.C. *et al.*, *Eur. J. Immunol.*, 23: 761-767 (1993) and Baggiolini, M. and C.A. Dahinden, *Immunology Today*, 15: 127-133 (1994)).

- 25 In one embodiment, an antagonist of CCR1 is identified by monitoring the release of an enzyme upon degranulation or exocytosis by a cell capable of this function. Cells expressing CCR1 can be maintained in a suitable medium under suitable conditions, and degranulation can be induced. The cells are contacted with an agent to be tested, and enzyme release can be assessed. The release of an enzyme into the medium can be detected or measured using a suitable assay, such as in an immunological assay, or biochemical assay for enzyme activity.

- 30 The medium can be assayed directly, by introducing components of the assay (e.g., substrate, co-factors, antibody) into the medium (e.g., before, simultaneous with or after the cells and agent are combined). The assay can also be performed on

medium which has been separated from the cells or further processed (e.g., fractionated) prior to assay. For example, convenient assays are available for enzymes, such as serine esterases (see e.g., Taub, D.D. *et al.*, *J. Immunol.*, 155: 3877-3888 (1995) regarding release of granule-derived serine esterases).

5 In another embodiment, cells expressing CCR1 are combined with a ligand of CCR1 or promoter of CCR1 function, an agent to be tested is added before, after or simultaneous therewith, and degranulation is assessed. Inhibition of ligand- or promoter-induced degranulation is indicative that the agent is an inhibitor of mammalian CCR1 function.

10 In a preferred embodiment, the antagonist of CCR1 function does not significantly inhibit the function of other chemokine receptors (e.g., CCR2, CXCR1, CCR3). Such CCR1-specific antagonists can be identified by suitable methods, such as by suitable modification of the methods described herein. For example, cells which do not express CCR1 (CCR1⁻) but do express one or more other chemokine
15 receptors (e.g., CCR2, CXCR1, CCR3) can be created or identified using suitable methods (e.g., transfection, antibody staining, western blot, RNase protection). Such cells or cellular fractions (e.g., membranes) obtained from such cells can be used in a suitable binding assay. For example, when a cell which is CCR1⁻ and CCR3⁺ is chosen, the CCR1 antagonist can be assayed for the capacity to inhibit the
20 binding of a suitable CCR3 ligand (e.g., RANTES, MCP-3) to the cell or cellular fraction, as described herein.

In another preferred embodiment, the antagonist of CCR1 function is an agent which binds to CCR1. Such CCR1-binding antagonists can be identified by suitable methods, for example, in binding assays employing a labeled (e.g., enzymatically
25 labeled (e.g., alkaline phosphatase, horse radish peroxidase), biotinylated, radio-labeled (e.g., ³H, ¹⁴C, ¹²⁵I)) antagonist.

In another preferred embodiment, the antagonist of CCR1 function is an agent which can inhibit the binding of a (i.e., one or more) CCR1 ligand to CCR1 (e.g., human CCR1).

30 In particularly preferred embodiment, the antagonist of CCR1 function is an agent which can bind to CCR1 and thereby inhibit the binding of a (i.e., one or more) CCR1 ligand to CCR1 (e.g., human CCR1).

Methods of Therapy

In one embodiment, the method of treating an inflammatory demyelinating disease comprises administering an effective amount of an (i.e., one or more) antagonist of CCR1 function to a subject in need thereof. Treatment includes
5 therapeutic or prophylactic treatment. According to the method, the severity of disease can be prevented or reduced in whole or in part.

In particular embodiments, the inflammatory demyelinating disease can be an acute inflammatory demyelinating disease, for example, acute disseminated encephalomyelitis, Guillain-Barre syndrome or acute hemorrhagic leukoencephalitis.

10 In other embodiments, the inflammatory demyelinating disease can be a chronic inflammatory demyelinating disease, for example, multiple sclerosis, chronic inflammatory demyelinating polyradiculoneuropathy.

In a preferred embodiment, the invention provides a method of treating (including therapeutic or prophylactic treatment) multiple sclerosis, comprising
15 administering an effective amount of an antagonist of CCR1 function to a subject in need thereof.

As discussed herein, the manifestation of MS is variable and the clinical course of MS can be grouped into four categories: relapsing-remitting, primary progressive, secondary progressive and progressive-relapsing. The method of the
20 invention can be used to treat MS which presents with each of the recognized clinical courses. Accordingly, an antagonist of CCR1 function can be administered to a patient with a progressive course of MS to retard or prevent the progression of neurological impairment. An antagonist of CCR1 function can also be administered to a subject with relapsing-remitting, secondary progressive or progressive-relapsing
25 MS as prophylaxis against relapse (e.g., an acute attack). For example, the antagonist of CCR1 function can be administered to a subject with relapsing-remitting MS during the remitting phase of the disease to inhibit (e.g., prevent, delay) relapse.

In another embodiment, the antagonist of CCR1 function is selected from the
30 group of molecules which can inhibit one or more functions of CCR1, for example,

certain small organic molecules, natural products, peptides, peptidomimetics and proteins, wherein said proteins are not chemokines or mutants or analogues thereof.

In another embodiment, the invention provides a method for treating (reducing or preventing) inflammatory demyelinating disease comprising administering an effective amount of an antagonist of CCR1 function and an effective amount of an (i.e., one or more) additional therapeutic agent to a subject in need thereof. The therapeutic benefit of an antagonist of CCR1 function and certain other therapeutic agents can be additive or synergistic when co-administered, thereby providing a highly efficacious treatment. Therapeutic agents suitable for administration with an antagonist of CCR1 function include, for example, antiviral agents (e.g., acyclovir, ganciclovir, famciclovir, penciclovir, valacyclovir, vidarabine, foscarnet, indinavir), antibacterial agents (e.g., antibiotics (e.g., erythromycin, penicillin, tetracyclin, ciprofloxacin, norfloxacin, flurazolidone, azithromycin, chloramphenicol), sulfonamides, quinalones), immunosuppressive agents, such as, calcineurin inhibitors (e.g., cyclosporin A, FK-506), IL-2 signal transduction inhibitors (e.g., rapamycin), glucocorticoids (e.g., prednisone, dexamethasone, methylprednisolone), nucleic acid synthesis inhibitors (e.g., azathioprine, mercaptopurine, mycophenolic acid) and antibodies to lymphocytes and antigen-binding fragments thereof (e.g., OKT3, anti-IL2 receptor). Additional therapeutic agents suitable for co-administration with an antagonist of CCR1 function include, for example, anti-inflammatory agents (e.g., aspirin, ibuprofen, naproxen, lysofylline), hormones (e.g., adrenocorticotrophic hormone (ACTH)), cytokines (e.g., interferons (e.g., IFN β -1a, IFN β -1b), Th2-promoting cytokines (e.g., IL-4, TGF- β)) and antibodies, such as antibodies that bind chemokines, cytokines or cell adhesion molecules (e.g., anti-CD11/CD18, anti-MIP 1 α) and copolymer 1.

The term "Th2-promoting cytokine", as used herein, refers to cytokines which inhibit the development/differentiation of Th1 cells and/or promote the development/differentiation of Th2 cells. Several "Th2-promoting cytokines" are known in the art and additional "Th2-promoting cytokines" can be identified using suitable methods (see, for example, Lingnau *et al.*, *J. Immunol* 161(9):4709-4718 (1998)).

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The particular co-therapeutic agent selected for administration with an antagonist of CCR1 function will depend on the type and severity of inflammatory demyelinating disease being treated as well as the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs. For example, in one embodiment, an antagonist of CCR1 function can be administered together with methylprednisolone or ACTH to treat a severe relapse of MS. In another embodiment, an antagonist of CCR1 function can be administered together with an immunosuppressive agent, IFN β -1a, IFN β -1b or copolymer 1 during the remitting phase of MS as prophylaxis against relapse. The skilled artisan will be able to determine the preferred co-therapeutic agent based upon these considerations and other factors.

The invention further relates to use of an antagonist of CCR1 function in therapy (including prophylaxis), for example, as described herein, and to the use of such an antagonist for the manufacture of a medicament for the treatment of inflammatory demyelinating disease (e.g., MS). The invention also relates to a medicament for the treatment of inflammatory demyelinating disease (e.g., MS) wherein said medicament comprises an antagonist of CCR1 function.

A "subject" is preferably a human, but can also be a mammal in need of veterinary treatment, e.g., domestic animals (e.g., dogs, cats, and the like), farm animals (e.g., cows, sheep, fowl, pigs, horses, and the like) and laboratory animals (e.g., rats, mice, guinea pigs, and the like).

An effective amount of the antagonist of CCR1 function can be administered to a subject to treat (reduce or prevent) inflammatory demyelinating disease. For example, an effective amount of the antagonist of CCR1 function can be administered before, during and/or after a demyelinating episode.

When co-administration of an antagonist of CCR1 function and an additional therapeutic agent is indicated or desired for treating inflammatory demyelinating disease, the antagonist of CCR1 function can be administered before, concurrently with or after administration of the additional therapeutic agent. When the antagonist of CCR1 function and additional therapeutic agent are administered at different times, they are preferably administered within a suitable time period to provide substantial overlap of the pharmacological activity (e.g., inhibition of CCR1

function, immunosuppression) of the agents. The skilled artisan will be able to determine the appropriate timing for co-administration of an antagonist of CCR1 function and an additional therapeutic agent depending on the particular agents selected and other factors.

- 5 An "effective amount" of a CCR1 antagonist is an amount sufficient to achieve a desired therapeutic and/or prophylactic effect, such as an amount sufficient to inhibit CNS inflammation, CNS demyelination and/or neurological dysfunction. For example an effective amount is an amount sufficient to inhibit a (i.e., one or more) function of CCR1 (e.g., CCR1 ligand-induced leukocyte migration, CCR1
10 ligand-induced integrin activation, CCR1 ligand-induced transient increase in the concentration of intracellular free calcium $[Ca^{2+}]_i$ and/or CCR1 ligand-induced secretion (e.g. degranulation) of proinflammatory mediators), and thereby, inhibit CNS inflammation, CNS demyelination and/or neurological dysfunction. An "effective amount" of an additional therapeutic agent (e.g., immunosuppressive
15 agent) is an amount sufficient to achieve a desired therapeutic and/or prophylactic effect (e.g., immunosuppression).

 The amount of agent (e.g., CCR1 antagonist, additional therapeutic agent) administered to the individual will depend on the characteristics of the individual, such as general health, age, sex, body weight and tolerance to drugs as well as the
20 degree, severity and type of inflammatory demyelinating disease. The skilled artisan will be able to determine appropriate dosages depending on these and other factors. Typically, an effective amount can range from about 0.1 mg per day to about 100 mg per day for an adult. Preferably, the dosage ranges from about 1 mg per day to about 100 mg per day.

- 25 The agent (e.g., CCR1 antagonist, additional therapeutic agent) can be administered by any suitable route, including, for example, orally in capsules, suspensions or tablets or by parenteral administration. Parenteral administration can include, for example, intramuscular, intravenous, subcutaneous, or intraperitoneal administration. The agent (e.g., CCR1 antagonist, additional therapeutic agent) can
30 also be administered orally (e.g., dietary), transdermally, topically, by inhalation (e.g., intrabronchial, intranasal, oral inhalation or intranasal drops) or rectally. Administration can be local or systemic as indicated. The preferred mode of

administration can vary depending upon the particular agent (e.g., CCR1 antagonist, additional therapeutic agent) chosen, however, oral or parenteral administration is generally preferred.

The agent (e.g., CCR1 antagonist, additional therapeutic agent) can be administered as a neutral compound or as a salt. Salts of compounds containing an amine or other basic group can be obtained, for example, by reacting with a suitable organic or inorganic acid, such as hydrogen chloride, hydrogen bromide, acetic acid, perchloric acid and the like. Compounds with a quaternary ammonium group also contain a counteranion such as chloride, bromide, iodide, acetate, perchlorate and the like. Salts of compounds containing a carboxylic acid or other acidic functional group can be prepared by reacting with a suitable base, for example, a hydroxide base. Salts of acidic functional groups contain a counteranion such as sodium, potassium and the like.

The antagonist of CCR1 function can be administered to the individual as part of a pharmaceutical composition for treating inflammatory demyelinating disease comprising a CCR1 antagonist and a pharmaceutically acceptable carrier.

Pharmaceutical compositions for co-therapy can comprise an antagonist of CCR1 function and one or more additional therapeutic agents. Alternatively, an antagonist of CCR1 function and an additional therapeutic agent can be components of separate pharmaceutical compositions which can be mixed together prior to administration or administered separately. Formulation will vary according to the route of administration selected (e.g., solution, emulsion, capsule). Suitable pharmaceutical carriers can contain inert ingredients which do not interact with the antagonist of CCR1 function and/or additional therapeutic agent. Standard pharmaceutical formulation techniques can be employed, such as those described in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA. Suitable pharmaceutical carriers for parenteral administration include, for example, sterile water, physiological saline, bacteriostatic saline (saline containing about 0.9% mg/ml benzyl alcohol), phosphate-buffered saline, Hank's solution, Ringer's-lactate and the like. Methods for encapsulating compositions (such as in a coating of hard gelatin or cyclodextran) are known in the art (Baker, *et al.*, "Controlled Release of Biological Active Agents", John Wiley and Sons, 1986).

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

EXAMPLES

Example 1

5 Materials and Methods

Animals

CCR1^{-/-} B6/129 mice (also referred to as CCR1 KO), which are homozygous for a target gene disruption of CCR1, were obtained from the laboratory of Dr. Craig Gerard and bred at Charles River Laboratories (Wilmington, MA) (Gerard, *et al.*, *J. Clin. Invest.*, 100(8):2022-2027 (1997)). Wild type CCR1^{+/+} B6/129 F1 animals were obtained from Taconic labs (Germantown, NY). Both male (>20 weeks of age) and female (6-8 weeks of age) animals were used for experiments.

MOG Peptide

The MOG(35-55) peptide (MEVGWYRSPFSRVVHLYRNGK, SEQ ID NO:1) described previously (Hilton, A.A. *et al.*, *J. Neurochem.*, 65: 309-318(1995)) was synthesized on an Applied Biosystems 433A Peptide Synthesizer (Applied Biosystems, Foster City, CA). The peptide which contains free amino and carboxyl termini was stored as a dessicate in 5 mg quantities at -80°C.

Immunization Protocol

20 Experiment 1

Young (6-8 week) female mice (CCR1^{-/-}, n = 10; CCR1^{+/+}, n = 10) were immunized as described previously (Bernard, C.C. *et al.*, *J. Mol. Med.*, 75: 77-88 (1997)). Briefly, animals were injected subcutaneously on the ventral abdomen with 100 µg MOG(35-55) peptide emulsified in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO) supplemented with 4 mg/ml (final concentration) *Mycobacterium tuberculosis* antigen (Difco, Detroit, MI). The total injectate volume was 100 µl divided between two sites. Mice were also injected with Pertussis toxin (Sigma, St. Louis, MO) in isotonic saline (150 ng, I.P.) on the day of immunization with MOG(35-55) peptide and two days later.

Experiment 2

Young (6-8 week) female mice (CCR1 $-/-$, $n = 8$; CCR1 $+/+$, $n = 8$) were immunized as described above in Experiment 1.

Experiment 3

- 5 Adult (> 20 weeks) male mice (CCR1 $-/-$, $n = 10$; CCR1 $+/+$, $n = 10$) were immunized as described above in Experiment 1.

Disease Scoring System

Mice were weighed and scored daily based upon the following scale:

- 0 = normal; 1 = loss of tail tone; 2 = posterior paresis; 3 = loss of righting response;
10 4 = tetraparesis, moribund. All data was analyzed using a two-tailed students' T test.

RESULTS

CCR1 $-/-$ mice are resistant to developing Experimental Allergic Encephalomyelitis (EAE).

- In each experiment, animals in both groups (CCR1 $+/+$ and CCR1 $-/-$) began
15 to develop disease symptoms by 10 days post-immunization, with the exception of adult male CCR1 $+/+$ mice which developed disease symptoms on day 15, and disease symptoms were most severe by about 16 days post-immunization. The incidence of disease in the CCR1 $-/-$ group was less than in the CCR1 $+/+$ group (Experiment 1: 8/10 vs. 10/10, Experiment 2: 5/8 vs. 8/8, Experiment 3: 1/10 vs.
20 8/10).

In each experiment, the mean clinical score in the CCR1 $-/-$ group was less than that of the CCR1 $+/+$ group at all time points. The difference in mean clinical score reached significance ($p < 0.05$) on days 11-23 and 36 of Experiment 1, days 10-28 of Experiment 2, and on day 17 of Experiment 3.

- 25 Following completion of each experiment, the maximal clinical score of each animal was determined, and the mean score \pm SD was derived for each group (ie, CCR1 KO and CCR1 $+/+$ control animals). The Students T test was utilized to determine if the difference between groups was statistically significant at the $p < 0.05$ level (Table 1).

Table 1 - Mean Maximal EAE Clinical Score of CCR1 KO and CCR1 +/+ Control Mice

Experiment	Gender	mean maximal clinical score \pm SD		p value
		CCR1 KO	B6/129	
1	female	1.9 \pm 1.3	3 \pm 0	0.015
2	female	1.6 \pm 1.4	3 \pm 0	0.015
3	male	0.3 \pm 0.95	1.2 \pm 0.9	0.04

Microscopic examination

Methods

On day fourteen of Experiment 2, CCR1 +/+ (n=4) and CCR1 -/- (n=3) animals were sacrificed, and selected tissues including cerebrum, cerebellum, brainstem and thoracolumbar spinal cord were removed and fixed in 10% neutral buffered formalin. Following routine processing, tissues were embedded in paraffin, sectioned at 6 μ m, stained with hematoxylin and eosin (H+E) and examined microscopically.

Results

CNS inflammation is less severe in CCR1 knockout mice.

All (4 of 4) CCR1 +/+ animals had lesions of variable severity and distribution in the examined tissues (some animals had one lesion at one site) as previously described (Slavin, *et al.*, *Autoimmunity*, 28:109-120 (1998)). Lesions consisted of focal to multifocal perivascular accumulation of lymphocytes in the space of Virchow-Robin. In some lesions, the lymphocytes extended into the adjacent neutrophil for a short distance resulting in axonal swelling and demyelination. The most severe lesions were observed in the spinal cords of two animals. In contrast, lesions were not observed in the examined tissues from CCR1 -/- animals. Thus, CNS inflammation in CCR1 -/- mice is less severe than in CCR1 +/+ control mice.

Example 2

Delayed Type Hypersensitivity Reaction

Mice were sensitized on the shaved abdomen with 25 μ l of 0.5% 2,4-dinitrofluorobenzene (DNFB, Aldrich, Milwaukee, WI) in a vehicle of 4:1 acetone:olive oil (Sigma, St. Louis, MO) on days 1 and 2 of the experiment. On day 5 of the experiment, animals were anesthetized with metaflane (Pitman/Moore, Mundelein, IL), ear thickness was measured (to 0.001 inch) and 20 μ l of 0.5% DNFB in a vehicle of 4:1 acetone:olive oil was applied to the top and bottom of each ear. On day 6, animals were euthanized and ears were measured again. Results were expressed as the change in ear thickness.

Results

Non-sensitized wild type B6/129 mice demonstrated minimal reaction to DNFB challenge and sensitized B6/129 mice demonstrated a vigorous reaction to DNFB challenge. In contrast, non-sensitized CCR1 $-/-$ mice had a modest reaction to DNFB, which was enhanced by prior sensitization. Thus, non-sensitized CCR1 $-/-$ mice are hyper-responsive to DNFB in comparison to wild type mice, but sensitized CCR1 $-/-$ mice respond normally.

Example 3

In Vitro Proliferation/Activation

Fourteen days after immunization with MOG peptide, CCR1 $-/-$ and wild type B6/129 mice were sacrificed and their spleens were removed. Splenocytes were cultured in 96-well plates at a concentration of 5×10^6 cell/ml in Dulbecco's minimal essential media (DMEM; Gibco) supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml), 10% heat inactivated bovine serum (Biowhittaker, Walkersville, MD) and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO).

For proliferation assays, serial dilutions (100, 50, 10, 5, 1 μ g/ml final concentration) of the MOG(35-55) peptide were added to the cultures at the time they were established. Control wells did not contain MOG(33-55) peptide. After 72 hours of culture, 3 H-thymidine was added to each well, and plates were cultured for a

further sixteen hours. The cultures were harvested and the quantity of ^3H -thymidine incorporated into the DNA of the cells was measured.

For cytokine assays, splenocytes were cultured in X-Vivo 20 serum free media media (Biowhittaker) containing MOG(35-55) peptide at 100 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$ or 1 $\mu\text{g/ml}$ final concentration. Control wells did not contain MOG(33-55) peptide. The cells were cultured for 40 hours, the culture supernatants were collected and the quantity of IL-2, IL-6 or IFN- γ in the supernatants was measured by ELISA.

Cytokine ELISAs

Quantitative ELISAs for murine IL-2, IL-6 and IFN- γ were performed using paired monoclonal antibodies (mAbs) specific for particular cytokines as recommended by the antibody supplier (Pharmingen, San Diego, CA). Briefly, 96-well microtiter plates (Dynatech, Chantilly, VA) were coated overnight at 4°C with 5 μg of purified rat anti-mouse cytokine capture antibody in 100 μl of carbonate buffer, pH 8.2. The plates were then washed three times with PBS containing 0.5% Tween 20 (polyethylenesorbate monolaurate; Sigma, St Louis, MO), blocked with 3% bovine serum albumin (Sigma, St. Louis, MO) in PBS, washed and incubated with culture supernatants or cytokine standards overnight at 4°C. The plates were then washed and incubated with a biotinylated rat anti-mouse cytokine detection antibody for 1 hour. The plates were washed, avidin-peroxidase was added and the plates were incubated for 20 minutes at room temperature. Color was developed with one component TMB (3,3',5,5'-tetramethyl-benzidine) reagent (KPL, Gaithersburg, MD).

Results

Splenocytes isolated from both CCR1 $^{-/-}$ and wild type B6/129 mice proliferated and produced IL-2, IL-6 and IFN- γ in response to MOG(35-55). There was no difference in the amount of antigen-specific proliferation of splenocytes from CCR1 $^{-/-}$ compared to splenocytes from wild type B6/129 mice when stimulated with MOG(35-55) (Figure 5A). Furthermore, there was no difference in the quantity of IL-2 (Figure 5B) or IL-6 (Figure 5D) produced in cultures of splenocytes from

-30-

CCR1^{-/-} or wild type B6/129 mice. In contrast, splenocytes from CCR1^{-/-} mice produced more IFN- γ than splenocytes from wild type B6/129 mice following stimulation with MOG(35-55)(Figure 5C). The results of the *in vitro* proliferation/activation assays and the DTH assay demonstrate that CCR1^{-/-} mice are
5 not generally immunosuppressed as a result of deletion of CCR1.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended
10 claims.

CLAIMS

What is claimed is:

1. A method of treating a demyelinating inflammatory disease comprising
administering to a subject in need thereof an effective amount of an antagonist
5 of CCR1 function.
2. The method of Claim 1 wherein said disease is an acute inflammatory
demyelinating disease.
3. The method of Claim 2 wherein said acute inflammatory demyelinating
disease is selected from the group consisting of acute disseminated
10 encephalomyelitis, Guillain-Barre syndrome and acute hemorrhagic
leukoencephalitis.
4. The method of Claim 3 wherein said disease is acute disseminated
encephalomyelitis.
5. The method of Claim 3 wherein said disease is Guillain-Barre syndrome
- 15 6. The method of Claim 3 wherein said disease is acute hemorrhagic
leukoencephalitis.
7. The method of Claim 1 wherein said disease is a chronic inflammatory
demyelinating disease.
8. The method of Claim 7 wherein said chronic inflammatory demyelinating
20 disease is selected from the group consisting of multiple sclerosis and chronic
inflammatory demyelinating polyradiculoneuropathy.
9. The method of Claim 8 wherein said disease is multiple sclerosis.

10. The method of Claim 1 wherein said antagonist of CCR1 function is selected from the group consisting of small organic molecules, natural products, peptides, proteins and peptidomimetics.
- 5 11. The method of Claim 10 wherein said antagonist of CCR1 function is a small organic molecule.
12. The method of Claim 10 wherein said antagonist of CCR1 function is a natural product.
13. The method of Claim 10 wherein said antagonist of CCR1 function is a peptide.
- 10 14. The method of Claim 10 wherein said antagonist of CCR1 function is a peptidomimetic.
15. The method of Claim 10 wherein said antagonist of CCR1 function is a protein.
- 15 16. The method of Claim 15 wherein said protein is an anti-CCR1 antibody or antigen-binding fragment thereof.
17. The method of Claim 1 further comprising administering to said subject an effective amount of an additional therapeutic agent selected from the group consisting of antiviral agents, antibacterial agents, immunosuppressive agents, cytokines and hormones.
- 20 18. The method of Claim 17 wherein said additional therapeutic agent is one or more immunosuppressive agents selected from the group consisting of calcineurin inhibitors, glucocorticoids, nucleic acid synthesis inhibitors, and antibodies which bind to lymphocytes or antigen-binding fragments thereof.

19. The method of Claim 18 wherein said immunosuppressive agent is a calcineurin inhibitor.
20. The method of Claim 19 wherein said immunosuppressive agent is cyclosporin A.
- 5 21. The method of Claim 18 wherein said immunosuppressive agent is a glucocorticoid.
22. The method of Claim 21 wherein said glucocorticoid is prednisone or methylprednisolone.
23. The method of Claim 17 wherein said additional therapeutic agent is a
10 cytokine.
24. The method of Claim 23 wherein said cytokine is an interferon.
25. The method of Claim 23 wherein said cytokine is a Th2-promoting cytokine.
26. The method of Claim 17 wherein said additional therapeutic agent is a hormone.
- 15 27. The method of Claim 26 wherein said hormone is adrenocorticotrophic hormone.

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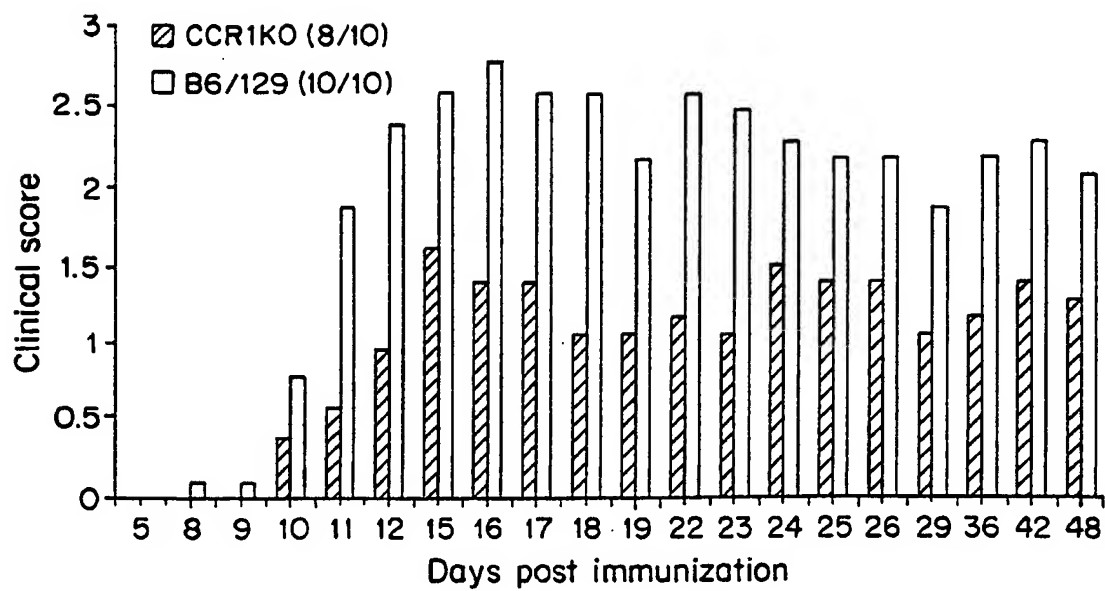


FIG. 1

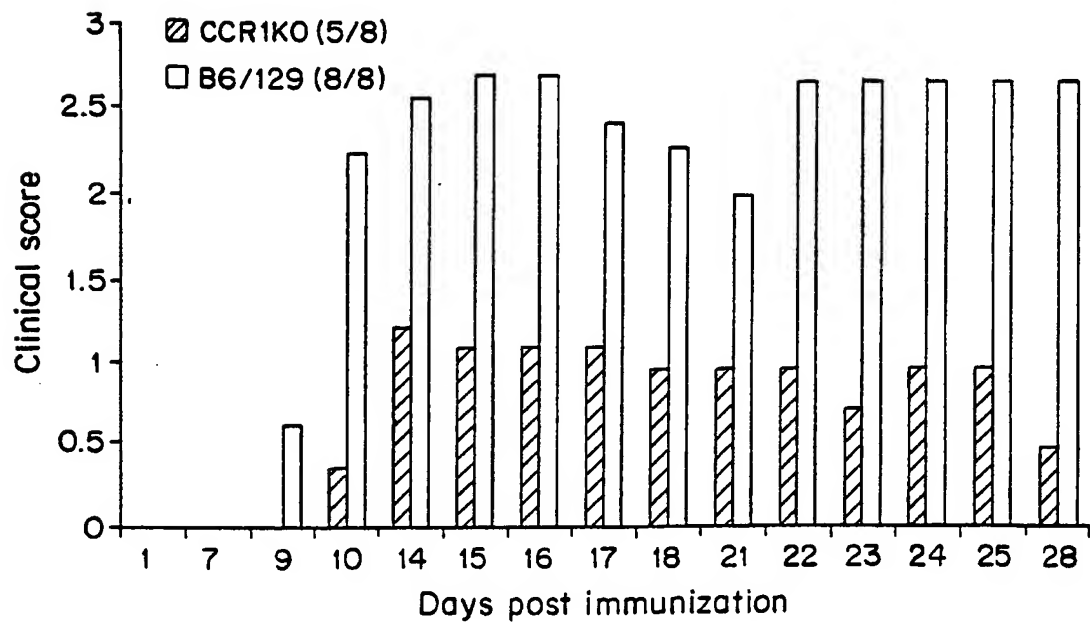
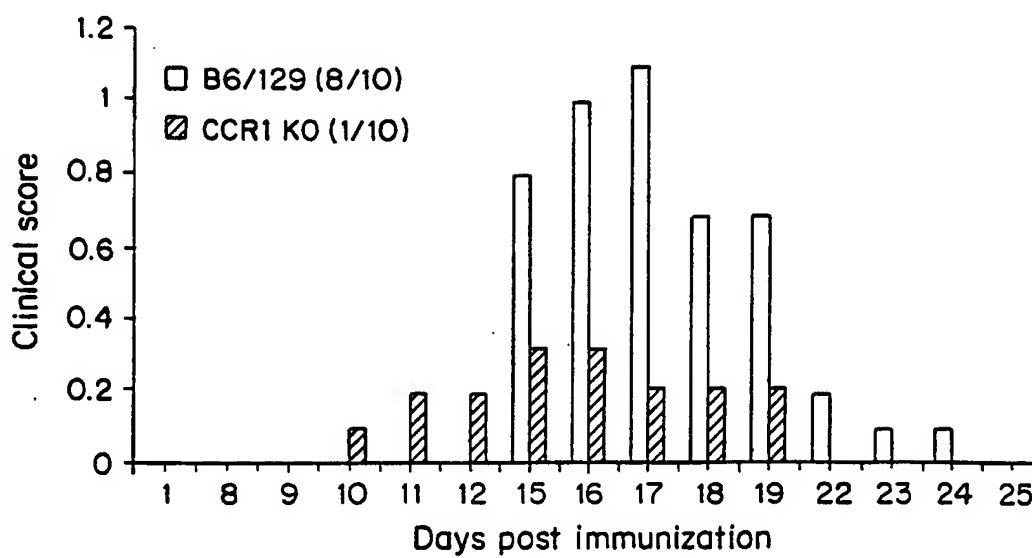
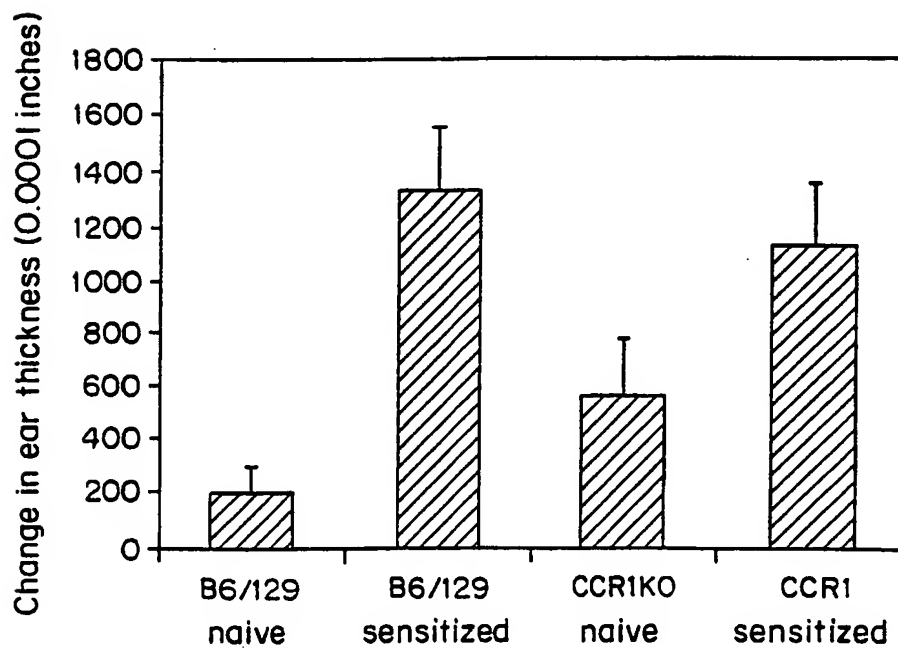


FIG. 2

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**FIG. 3****FIG. 4**

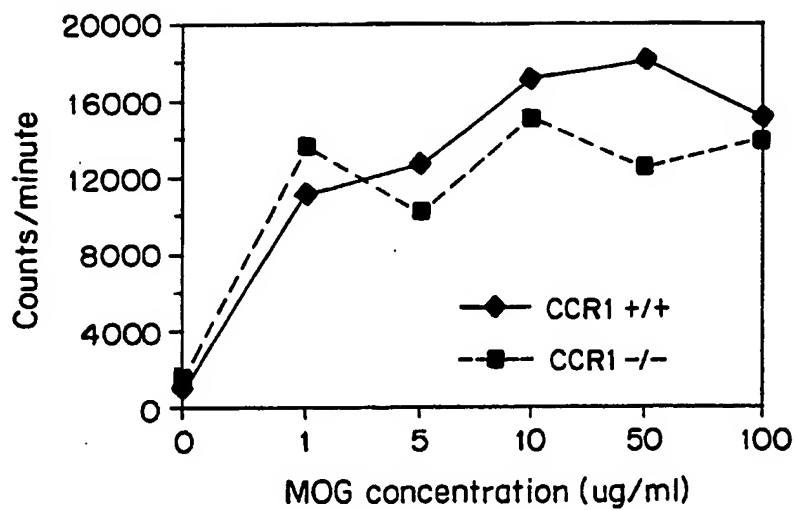


FIG. 5A

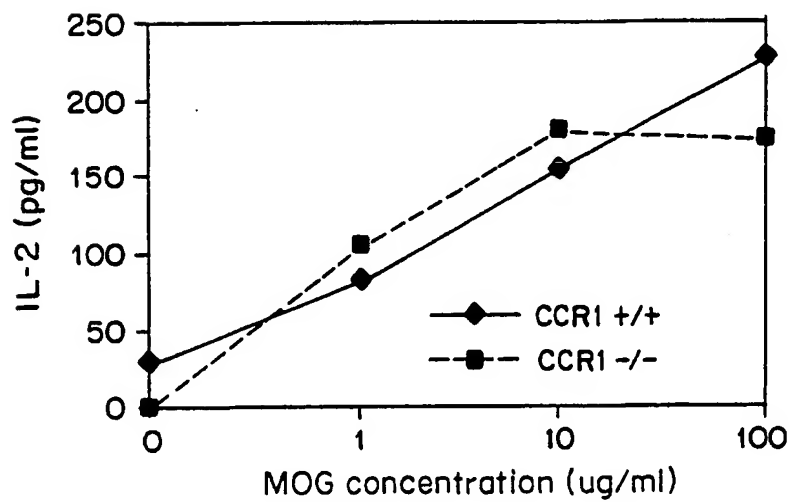


FIG. 5B

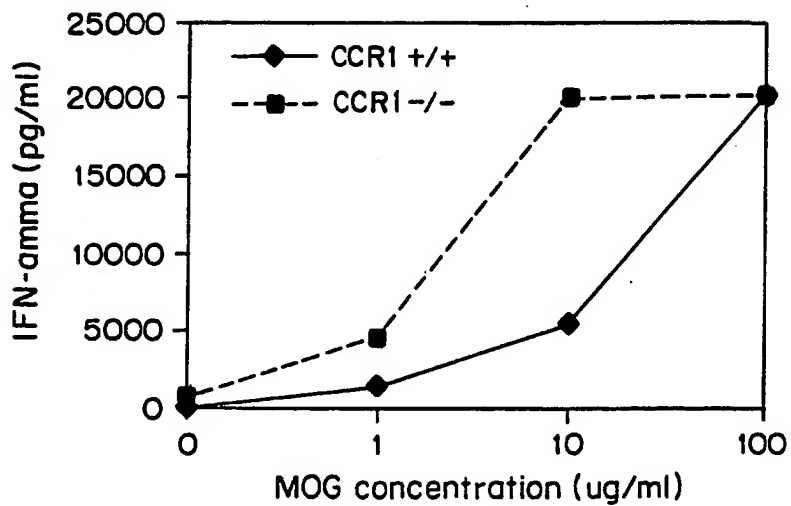


FIG. 5C

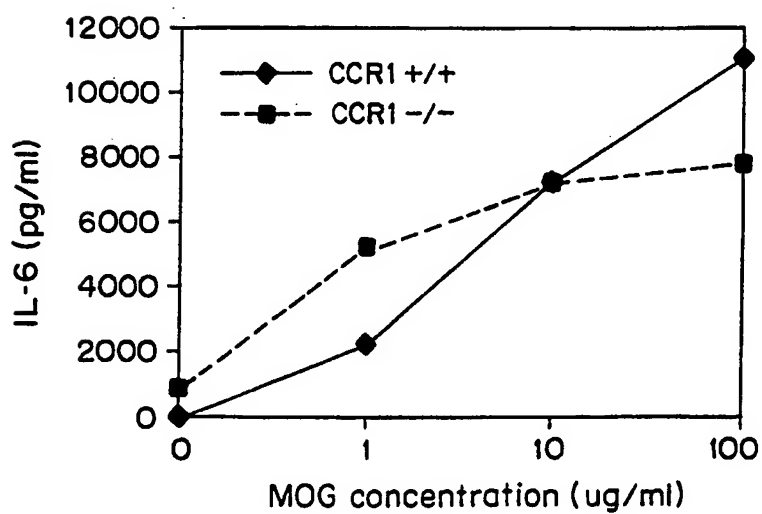


FIG. 5D

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/02124

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 97 24325 A (TAKEDA CHEMICAL INDUSTRIES LTD ;KATO KANEYOSHI (JP); YAMAMOTO MITS) 10 July 1997 (1997-07-10) cited in the application * see in particular claims 1,41; page 2, lines 4-24; pages 202-204 (Test example 1) *</p>	1,7-11
X	<p>--- HESSELGESSER ET AL.: "Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor" J. BIOLOGICAL CHEMISTRY, vol. 273, no. 25, 19 June 1998 (1998-06-19), pages 15687-15692, XP000887384 cited in the application *see in particular the abstract; Table 2; Figure 2; last paragraph of the discussion *</p>	1,7-11
X	<p>--- DATABASE WPI Section Ch, Week 199748 Derwent Publications Ltd., London, GB; Class B02, AN 1997-521868 XP002139271 & JP 09 249570 A (TAKEDA CHEM IND LTD), 22 September 1997 (1997-09-22) abstract</p>	1,7-11
X	<p>--- DATABASE WPI Section Ch, Week 199804 Derwent Publications Ltd., London, GB; Class B03, AN 1998-035793 XP002139272 & JP 09 249566 A (TAKEDA CHEM IND LTD), 22 September 1997 (1997-09-22) abstract -----</p>	1,7-11

INTERNATIONAL SEARCH REPORT

In. ational application No.
PCT/US 00/02124

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-10 in part, 11, 17-27 in part

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-10 in part; 11; 17-27 in part

Method of treating a demyelinating inflammatory disease comprising an antagonist of CCR1 function wherein the antagonist is a small organic molecule

2. Claims: 1-10 in part; 12; 17-27 in part

Method of treating a demyelinating inflammatory disease comprising an antagonist of CCR1 function wherein the antagonist is a natural product

3. Claims: 1-10 in part; 13; 17-27 in part

Method of treating a demyelinating inflammatory disease comprising an antagonist of CCR1 function wherein the antagonist is a peptide

4. Claims: 1-10 in part; 15,16; 17-27 in part

Method of treating a demyelinating inflammatory disease comprising an antagonist of CCR1 function wherein the antagonist is a protein

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/02124

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